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Susceptibility of *Naegleria fowleri* to Δ^9-Tetrahydrocannabinol

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Growth of the pathogenic amoeboflagellate *Naegleria fowleri* is inhibited by Δ^9-tetrahydrocannabinol (Δ^9-THC). Δ^9-THC is amoebostatic at 5 to 50 μg/ml. Δ^9-THC prevents enflagellation and encystment, but does not impair amoeboid movement. Calf serum at 10 and 20% (vol/vol) reduces the antiamoeba activity of Δ^9-THC. Only 1-methoxy Δ^8-tetrahydrocannabinol, of 17 cannabinoids tested, failed to inhibit growth of *N. fowleri*. Antinaeglerial activity was not markedly altered by opening the pyran ring, by converting the cyclohexyl ring to an aromatic ring, or by reversing the hydroxyl and pentyl groups on the benzene ring. Δ^9-THC prevented the cytopathic effect of *N. fowleri* on African green monkey (Vero) cells and human epithelioma (HEp-2) cells in culture. Δ^9-THC afforded modest protection to mice infected with *N. fowleri*.

Naegleria fowleri is the etiological agent of primary amoebic meningoencephalitis in humans (6, 10). To date, amphotericin B is the most effective antinaeglerial drug in vitro, is able to protect mice infected with *N. fowleri* (9), and appears to alter favorably the outcome of primary amoebic meningoencephalitis in humans (3). Because of the serious nature of naeglerial infection in humans (7) and the toxicity of amphotericin B (2, 17), a satisfactory chemotherapeutic agent for the treatment of primary amoebic meningoencephalitis in humans is yet to be developed.

Δ^9-Tetrahydrocannabinol (Δ^9-THC) has been reported to inhibit the growth of several protozoa, including *Dictyostelium discoideum* (5) and *Tetrahymena pyriformis* (14). In addition, Δ^9-THC has been reported to retard the proliferation of tumor cells in vivo (16) and in vitro (18). Because Δ^9-THC accumulates in the central nervous system (13) and *N. fowleri* invades the brain (8), the cannabinoids merit examination as potential drugs for the treatment of primary amoebic meningoencephalitis.

MATERIALS AND METHODS

N. fowleri LEE was grown in Nelson medium, consisting of Page saline (0.12 g of NaCl, 0.142 g of Na_2HPO_4, 0.136 g of KH_2PO_4, 0.004 g of $MgSO_4 \cdot 7H_2O$, and 0.004 g of $CaCl_2$ per liter of distilled water) supplemented with 0.1% (wt/vol) Pan Meade liver digest (Harrison and Grosfield, Bronxville, N.Y.), 0.1% (wt/vol) glucose, and 2 or 5% (vol/vol) calf serum (GIBCO, Grand Island, N.Y.). Tissue culture flasks (25-cm^2 size; Falcon Plastics, Oxnard, Calif.) containing 10 ml of medium were inoculated to give 1×10^4 to 5×10^4 amoebae per ml and were incubated for 96 h at 37°C (1).

Δ^9-THC and selected cannabinoids were obtained from the National Institute on Drug Abuse (Rockville, Md.) and dissolved in Emulphor-ethanol (EL-620; GAF Corp., New York, N.Y., and ethanol, 1:1) at a concentration of 20 mg/ml. This preparation was diluted in Page saline as appropriate.

To evaluate the effect of Δ^9-THC and related cannabinoids on growth, amoebae from rapidly growing cultures were inoculated into growth medium (supplemented with 2% calf serum) to give 1×10^4 to 5×10^4 trophozoites per ml. The cannabinoids were added, and the cultures were incubated at 37°C. When indicated, cells were suspended by vigorous agitation or by chilling the culture to 5°C for 10 min; 1-ml samples were removed for counting, and cultures were returned to 37°C. For cell counts, 0.2 ml of the culture was added to 9.8 ml of an electrolyte solution (consisting of NaCl, 0.4% [wt/vol] and Formalin, 0.5% [vol/vol] in distilled water). Cell counts were made using an electronic cell counter (Coulter Counter model Z_B; Coulter Electronics, Inc., Hialeah, Fla.).

The capability of Δ^9-THC to prevent the cytopathic effects of *N. fowleri* on African green monkey (Vero) cells and human epithelioma (HEp-2) cells in culture was assessed. The two established cell cultures were grown in Eagle basal medium with Earle balanced salt solution, supplemented with 10% fetal calf serum (Flow Laboratories, Rockville, Md.) as described previously (4, 15). Antibiotics were not added. *N. fowleri* at a final population of 10^4 to 10^5 amoebae per ml was added to Vero or HEp-2 cultures with confluent cell monolayers. Diluent of Δ^9-THC was added simultaneously when indicated, and the cell cultures were incubated at 37°C for 48 h. Cytopathogenicity was scored visually with the aid of a compound light microscope.

To evaluate the effect of Δ^9-THC on the susceptibility of mice to naeglerial infection, BALB/c male mice (50) were inoculated intranasally with 1 50% lethal dose of rapidly growing *Naegleria*. One group served as the infected but untreated control; the other group was given 50 mg of Δ^9-THC per kg intraperitoneally on 4 consecutive days. The mice were observed daily for 3 weeks.

RESULTS

The growth of *Naegleria fowleri* was retarded by 5 and 10 μg of Δ^9-THC per ml and was markedly inhibited by 20 μg of Δ^9-THC per ml (Fig. 1). Emulphor-ethanol at the concentration needed to deliver 20 μg of Δ^9-THC per ml had little effect on the growth of *N. fowleri*. In contrast, 2 mg of Δ^9-THC per ml failed to inhibit the growth of *Mycobacterium marinum* 437 or *Saccharomyces cerevisiae* ATCC 9763, nor did 400 μg/ml (maximum concentration tested) inhibit *Cryptococcus laurentii* 9-389. Δ^9-THC was amoebostatic at 20 μg/ml, but amoebicidal at greater concentrations (50 to 100 μg/ml). After prolonged incubation in medium containing 20 μg of Δ^9-THC per ml the amoebae began to proliferate, but with a significantly extended generation time. However, this population of amoebae possessed the same susceptibility to the drug as those that had not been exposed to the drug. Attempts to select for variants of *N. fowleri* resistant to Δ^9-THC have not been successful.

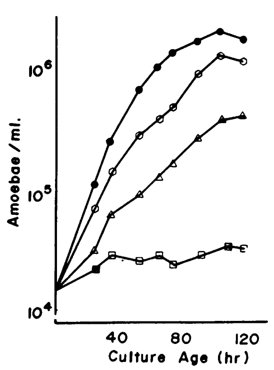


FIG. 1. *Growth of N. fowleri. Diluent only* (●); *5 μg of Δ^9-THC per ml* (○); *10 μg of Δ^9-THC per ml* (Δ); *and 20 μg of Δ^9-THC per ml* (□).

Most *N. fowleri* amoebae exposed to 20 μg of Δ^9-THC per ml became rounded and detached from the surface. The viable spherical cells, whether attached or free, did not differentiate into flagellates or form cysts. Those cells that remained attached to the surface displayed essentially normal amoeboid mobility.

Calf serum added to give 1% final concentration failed to support growth of *N. fowleri* (data not presented). Serum concentrations greater than 10% inhibited growth. The inhibitory effect of elevated concentrations of calf serum could be reduced by dialyzing the serum. Δ^9-THC at concentrations of 10 and 20 μg/ml inhibited growth of *N. fowleri* in medium containing 5% serum but not in medium containing 10% serum or 20% dialyzed serum (Table 1). Ca^{2+} (3.6×10^{-5} to 3.6×10^{-3} M) and Fe^{2+} (10^{-6} to 10^{-4} M) had little effect on the growth of *N. fowleri* and did not alter the susceptibility of *N. fowleri* to Δ^9-THC.

A number of cannabinoids inhibited the growth of *N. fowleri*. Of 17 cannabinoids tested, only 1-methoxy Δ^8-tetrahydrocannabinol failed to inhibit the proliferation of *N. fowleri* at 10 to 20 μg/ml (Table 2). The remainder of the cannabinoids may be grouped as those more active than Δ^9-THC and those with the same activity as Δ^9-THC. Two cannabinoids somewhat more active than Δ^9-THC were 11-hydroxy Δ^8-tetrahydrocannabinol and 9-*nor*-9-hydroxycannabinol. Both of these molecules differ from the parent Δ^9-THC at two sites in ring III (Fig. 2). The inactive 1-methoxy Δ^8-THC differs from the active Δ^8-THC at only one site in ring I. Cannabinoids with an aromatic ring III retained antinaeglerial activity. Either a methyl group or a hydroxyl group could occupy position 11 of ring III. The antinaeglerial activity of the cannabinoids was not markedly altered by opening of ring II or by the removal of the methyl groups from ring II. The antinaeglerial activity was not markedly altered when the hydroxyl group and pentyl chain of ring I were reversed. However, if the hydroxyl group was substituted with a methoxy group, the antinaeglerial activity was lost.

N. fowleri was cytopathic for Vero cells and

TABLE 1. *Effect of serum concentration on the antinaeglerial activity of Δ^9-THC*

Treatment	Amoebae per ml of growth medium containing serum concn:[a]				
	5%	5% dialyzed[b]	10%	20%	20% dialyzed[b]
Untreated	1.4×10^6	1.1×10^6	1.4×10^6	1.7×10^5	1.3×10^6
Diluent	1.2×10^6	ND[c]	1.4×10^6	1.7×10^5	1.2×10^6
10 μg of Δ^9-THC per ml	6.1×10^5	5.2×10^6	1.3×10^6	1.3×10^5	1.1×10^6
20 μg of Δ^9-THC per ml	1.7×10^5	ND	9.1×10^5	1.2×10^5	9.0×10^5

[a] Inoculum: 5×10^4 amoebae per ml; cultures were incubated for 3 days.
[b] Dialyzed calf serum.
[c] ND, Not done.

TABLE 2. *Inhibitory effect of different cannabinoids on the proliferation of N. fowleri*

Cannabinoid	Amoebae (× 10^3) per ml on day 3 at dose level:		
	5 µg/ml	10 µg/ml	20 µg/ml
Diluent	1,296	1,054	914
1-Methoxy Δ^8-Tetrahydrocannabinol[a]	1,073	922	823
Δ^9-Tetrahydrocannabinol[b]	692	220	106
Δ^8-Tetrahydrocannabinol[b]	652	310	108
(±) 9-*nor*-9β-Hydroxy hexahydrocannabinol[c]	1,131	499	165
9-*nor*-Cannabinol[c]	401	301	184
9-*nor*-9β-Hydroxy Δ^{10}-tetrahydroxycannabinol[c]	645	299	114
11-Hydroxy Δ^9-tetrahydrocannabinol[b]	232	114	34
11-Hydroxy Δ^8-tetrahydrocannabinol[b]	261	70	39
8β-Hydroxy Δ^9-tetrahydrocannabinol[c]	572	227	8.5
Abnormal cannabinol[d]	948	493	36
(±) 11-Hydroxy Δ^{6a-10a}-tetrahydrocannabinol[c]	411	215	29
11-Methoxy Δ^8-tetrahydrocannabinol[c]	709	261	49
Cannabinol[b,d]	555	207	64
Cannabidiol[b,d]	350	137	56
9-*nor*-9-Hydroxycannabinol[c]	466	61	37
6,6,9-Tri *nor*-6-oxo Δ^{6a-10a}-tetrahydrocannabinol[c]	471	114	25
(±) 9-*nor*-9-Hydroxy Δ^{6a-10a}-tetrahydrocannabinol[c]	804	403	32

[a] Prepared by William Vincek, Medical College of Virginia.
[b] Kindly supplied by the National Institute on Drug Abuse.
[c] From the laboratory of Everette May, National Institute of Arthritis, Metabolism and Digestive Diseases.
[d] Kindly supplied by Raj K. Razdan, SISA Inc.

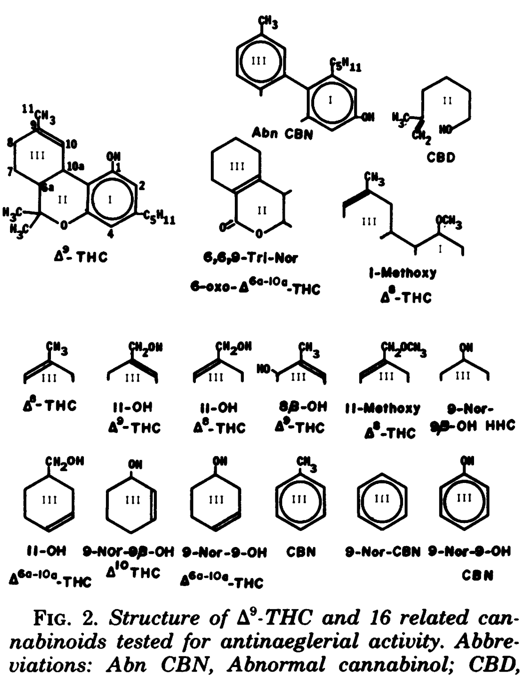


FIG. 2. *Structure of Δ^9-THC and 16 related cannabinoids tested for antinaeglerial activity. Abbreviations: Abn CBN, Abnormal cannabinol; CBD, cannabidiol; CBN, cannabinol; OH, hydroxy; HHC, hexahydrocannabinol.*

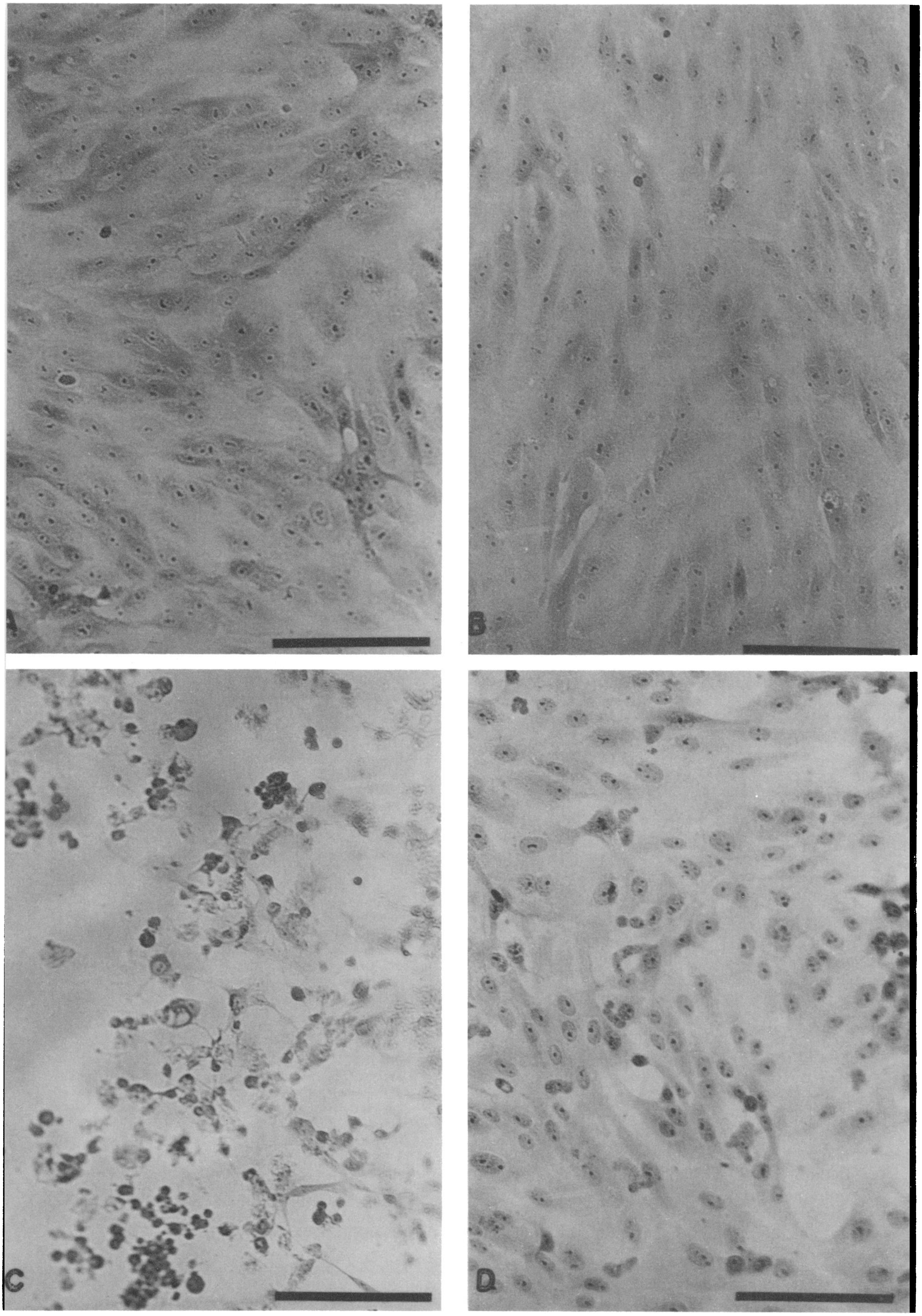
HEp-2 cells in culture. At 10^5 amoebae per ml, the cultured mammalian cells were destroyed within 24 h; with 10^4 amoebae per ml, 48 h was required. Growth of *N. fowleri* was very limited in the mammalian cell cultures and in the cell culture medium. The amoebae displayed normal morphology and were actively mobile in mixed culture with the mammalian cells. Δ^9-THC at 20 to 50 µg/ml protected Vero and HEp-2 cells from the cytopathic action of *N. fowleri* (Fig. 3). Above 50 µg/ml, Δ^9-THC was cytotoxic for the mammalian cell monolayer.

Δ^9-THC afforded some protection in mice to naeglerial infection (Fig. 4). The protection afforded by Δ^9-THC involved both extension of life and 25% fewer deaths. Similar results were obtained with mice infected with *N. fowleri* intravenously.

DISCUSSION

Δ^9-THC inhibits growth of several protozoa (5, 14) and mammalian cells (12, 18), but does not inhibit growth of selected yeast or bacteria. Δ^9-THC prevents enflagellation and encystment by *N. fowleri*, but not amoeboid mobility, indicating that some energy generating and transfer systems are functional, but that macromolecular

FIG. 3. *Protection of Vero cell cultures from the cytopathic effect of N. fowleri by 20 µg of Δ^9-THC per ml. (A) Normal Vero cells; (B) uninfected Vero cells, plus 20 µg of Δ^9-THC per ml; (C) Vero cell monolayers infected with N. fowleri; (D) Vero cell monolayer infected with N. fowleri, with 20 µg of Δ^9-THC per ml added simultaneously. After 24 h, the cultures were drained, rinsed, fixed, and stained with Gomori modified trichrome. The scale markers denote 50 µm.*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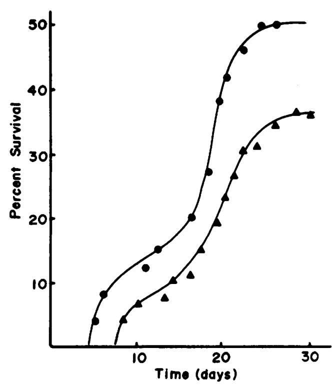

FIG. 4. *Survival of mice infected with N. fowleri intranasally on day 0 and treated with diluent only* (Δ) *or 50 mg of* Δ^9*-THC per kg daily on days 1 to 4* (●). Δ^9*-THC was administered intraperitoneally.*

synthesis is inhibited directly or indirectly. Δ^9-THC binds to proteins, and the antinaeglerial activity of the Δ^9-THC-protein complex is reduced. Both serum concentration and inoculum size markedly influenced the degree of inhibition observed with lower (5 to 20 μg/ml) concentrations of Δ^9-THC. *N. fowleri* will not grow in standard mammalian cell culture media. Several mammalian cell culture media can support naeglerial growth when diluted fivefold or more. The inhibition of growth with higher concentrations (20%, vol/vol) of undialyzed serum appears to be due to the toxicity of inorganic salts; however, calcium salts did not markedly affect growth of naegleria or the action of Δ^9-THC on naegleria. The reported capacity of calcium to alter the effects of Δ^9-THC on continuous cell lines (12) does not seem to be a general effect.

The cannabinoid structure can be substantially altered without loss of antinaeglerial activity. Methoxylation of the hydroxyl group of the phenolic ring drastically reduced its activity. The essential role of the phenolic group might indicate that an interaction between cannabinoids and iron-containing molecules is involved in its mechanism of action, but added inorganic iron salts had no effect on the inhibitory activity of Δ^9-THC. Cannabinoids have a wide variety of biological effects, including psychotomimetic, immunosuppressant, antitumor, and antimicrobial activities (14, 18). These diverse activities are not absolutely linked, because 1-methoxy Δ^8-THC is an effective immunosuppressant agent, but has little antinaeglerial and little central nervous system activities. Abnormal cannabinol has little central nervous system and immunosuppressant activity, but has antinaeglerial activity. These varied activities, however, do not necessarily mean that the molecular mechanisms of action of various cannabinoids are different, but that they may differ in solubility, distribution, permeation, availability, and metabolism. Clearly the action of cannabinoids is not limited to highly specialized nerve cells; thus the mechanism of action of cannabinoids may be best pursued in a microbial system.

Δ^9-THC prevented the cytopathic effect of *N. fowleri* on Vero or HEp-2 cells in culture. The cytopathic effect on the monolayer is presumably the result of a cytotoxic phospholipolytic factor and actual phagocytosis (11). Δ^9-THC was not amoebicidal under our experimental conditions, so the basis of the protection afforded by Δ^9-THC is not known. Δ^9-THC may impair the synthesis or release of a cytopathic phospholipase.

Δ^9-THC provided only modest protection for mice infected intranasally or intravenously with *N. fowleri*. Δ^9-THC is not the most active cannabinoid in vitro, so a more effective response in vivo may be achieved with other cannabinoids.

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